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Structural Elements and Neuropharmacological Features Involved in the Insecticidal Properties of a Scorpion Neurotoxin: A Multidisciplinary Approach

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Appendix VI. BARD Cover Page for Scientific Reports

To be sent to BARD Office in Israel:

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Project's starting date: August, 1995

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Principal Investigator

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Institution's Authorizing Official

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Abstract

Integrated pest management in modern crop protection requires the use of chemical or biological insecticides in many instances. Nontheless, the use non-selective chemical insecticides poses risks to the environment and livestock and consequently urgent need exists for safer alternatives, which target insects more specifically. Scorpions produce anti-insect selective polypeptide toxins that are biodegradable and not toxic to warm-blooded animals. Therefore, mobilization of these substances into insect pest targets is of major interest. Moreover, clarification of the molecular basis of this selectivity may provide valuable information pertinent to their receptor sites and to the future design of peptidomimetic anti-insect specific substances. These toxins may also be important for reducing the current overuse of chemical insecticides provided they have a synergistic effect with conventional pesticides. All of these objectives were addressed in this research. A direct approach for plant protection was the mobilization of toxins into target pests using baculoviral vectors. The other approach was to develop a suitable system enabling the elucidation of the toxin bioactive site, which would enable design of insecticidal peptidomimetics. In parallel, the mode of action and synergistic effects of scorpion insecticidal toxins, were studied at the sodium channel receptor site. All the above approaches show great promise and clearly indicate that scorpion insecticidal toxins may provide powerful means in insect pest control.

Objectives of the original research

The objectives of this project were to elucidate the structural elements, which constitute the toxic site and confer anti-insect preference of LqhαIT, and to study the mode of action and synergistic effects among pharmacologically distinct toxins and with pyrethroids.

Relevant data, methodology, results and discussion

The objectives delineated in the proposal have been met in both laboratories. Part of the data was published (see hereafter) and therefore is described in the report only in general terms. The part, which is mostly still unpublished, is described in detail.

Elucidation of the bioactive site of LqhαIT (Tel-Aviv):

We have developed an efficient bacterial expression system for production of milligram amounts of functional scorpion neurotoxins (**Zilberberg et al., 1996; Turkov et al., 1997**). This achievement was crucial for any further attempt to study structure-activity relationship and also enabled to determine the solution structure of LqhαIT by 2D-^{1}H-NMR (**Tugarinov et al., 1997**). More than 40 LqhαIT mutants have been generated and each was produced as non-soluble inclusion bodies in *E. coli*, denatured and folded *in vitro*, and the functional isoform purified by C_{18}-RP-HPLC. All toxin variants were analyzed by toxicity assay on blowfly larvae as a quick and direct measure for activity. Binding assays to a cockroach neuronal membrane preparation were used as a measure for direct activity at the receptor site, and possible structural alterations were assessed by circular dichroism spectroscopy. This thorough analysis highlighted the C-terminal region, the five-residue turn, and surrounding residues as the structural elements constituting the bioactive surface. This part is described in detail in **Zilberberg et al., 1997**. This study has also highlighted residues important for spatial arrangement or for the formation of a polar electrostatic potential important for toxicity. We are today at the stage where reciprocal alterations in the toxin active site and in the, yet unknown, receptor binding site of the channel, will complete the identification of

the interacting surface. Thus, a functional recombinant sodium channel is now required and initial steps toward this goal, taken in Riverside, are described hereafter. Despite this limitation, the above results motivated us to attempt active site exchange between LqhαIT (the most insecticidal toxin among the alpha group) and Lqh II (a homolog of AaH II, the strongest anti-mammalian scorpion α-neurotoxin). Two major structural motifs involved in the bioactive surface of LqhαIT and varying slightly in AaH II, were engineered in a reciprocal manner. Biological assays of the recombinant chimera-like toxins has shown inversion in phylogenetic preference (**not yet published**). This experiment indicates that these toxins are flexible and amenable for further engineering. It also strengthens our suggestion as to the location of the toxic site.

Increasing the insecticidal efficacy of baculoviruses by their engineering to express scorpion insecticidal toxins (Volcani Center & Tel-Aviv):

In collaboration with N. Chejanovsky and E. Geshburg, a joint Ph.D. student at the Volcani center, we have shown that engineering of AcNPV (*Autographa californica polyhedrovirus*) to express LqhαIT increased considerably its insecticidity to *Spodoptera littoralis* larvae (**Chejanovsky et al., 1995**). During the current grant period, we have extended this study to the excitatory and depressant anti-insect selective toxins derived from the Israeli yellow scorpion (*Leiurus quinquestriatus hebraeus*). Similarly to the construction with LqhαIT, each of the cDNA clones (including their original sequence encoding for a leader peptide) have been placed under the control of the polyhedrin promoter of AcNPV and the recombinant viruses were tested on noctuid larvae. The results indicated clearly an advantage for the depressant toxin, LqhIT2, over the excitatory toxin, LqhIT1 (equivalent of the heralded AaHIT: **Maeda et al., 1991; Stewart et al., 1991; McCutchen et al., 1991**) with an improvement of up to 50% in the time to kill of pest larvae (**Gershburg et al., 1998**). These results encouraged us to study viral promoters and means for further improvement of the insecticidal efficacy achieved thus far (**unpublished results**).

Mode of action at the sodium channel site (Riverside):

During the previous BARD award (July 1992-June, 1995) we investigated the behavioral synaptic effects of the insect-selective alpha-scorpion toxin, LqhαIT. We showed that LqhαIT prolongs the duration of action potentials in the presynaptic nerve terminal. To further investigate actions of LqhαIT on crop pests, we have developed voltage clamp techniques for studies of sodium channels in central neurons of the tobacco budworm, *Heliothis virescens* and the housefly, *Musca domestica*. This was an important step, since protein toxin genes now are being incorporated into recombinant viruses for agricultural use (**Chejanovsky et al., 1995; Gershburg et al., 1998**).

We also have begun to evaluate potential synergistic interactions between scorpion toxins and conventional insecticides. We first examined modification of *H. virescens* sodium channels by the synthetic pyrethroid permethrin and then asked whether co-application or pretreatment with scorpion toxins (LqhαIT, AaIT) could enhance pyrethroid potency. To put this question in context, it has been shown that joint application of an engineered viral insecticide expressing a scorpion toxin and a pyrethroid enhances the speed of kill in synergistic manner (**McCutchen et al., 1997**). We sought to verify this observation at the molecular level by examining the joint actions of permethrin and scorpion toxin at the level of *H. virescens* sodium channels.

A third objective was to test whether sodium channels of insecticide-resistant insects are more sensitive to scorpion toxins than susceptible insects, which was implied by the work of McCutchen et al. (**1997**). Specifically, we sought to determine whether scorpion toxins are more potent against sodium channels of pyrethroid-resistant *H. virescens* and *M. domestica*.

1. Modification of *H. virescens* sodium channels by LqhαIT: We successfully developed a method for short-term culture of *H. virescens* neurons to allow recording of sodium currents using the whole-cell patch clamp technique. Exposure of these neurons to LqhαIT (40 nM) resulted in a dramatic slowing of sodium

channel inactivation (**Fig. 1A, B**). This effect was more obvious when steady-state sodium currents were plotted as a function of test potentials (**Fig. 1C**). LqhαIT also caused an approximate 50% increase of the amplitude of evoked sodium currents at all test potentials (**Fig. 1B and D**).

The elevation of peak sodium currents can be explained by 1) modification of sodium channel activation and/or 2) inhibition of channel inactivation. First, we tested whether LqhαIT modifies voltage-dependent sodium conductance (**Hille, 1992**). As can be seen in **Fig. 1E**, voltage-dependent activation of sodium channels was not modified, but steady-state inactivation was dramatically inhibited (**Fig. 1F**). These results are consistent with a previous report that the major effect of LqhαIT slows sodium channel inactivation in cockroach giant axons (**Eitan et al., 1990**). The action of LqhαIT on the sodium channel inactivation process proved useful in the construction of dose-response curves. The elevation of steady-state sodium current was used to quantify concentration-dependent modification caused by LqhαIT in pyrethroid-susceptible and -resistant *H. virescens* neurons (see below).

2. Modification of H. virescens sodium channels by permethrin: Little information is available regarding modification of pest insect sodium channels by pyrethroids. We wanted to characterize the actions of permethrin on sodium channels in *H. virescens* neurons, then examine joint actions of permethrin with scorpion toxins. Our specific objectives were: 1) to quantify the concentration-dependence pyrethroid action on sodium channels, 2) to examine possible potentiation of pyrethroid action by an insect-selective scorpion toxin, and 3) to compare the potency of LqhαIT against sodium channels in susceptible and resistant insects. Permethrin exposure dramatically inhibited sodium channel deactivation {**Fig. 2A**, I_{Na}(ss-tail)} following test pulses. Such prolonged tail currents often persisted for several seconds. The elevation of steady-state tail currents was test potential dependent (**Fig. 2C**) at lower test potentials. At more positive test potentials, this elevation was saturated. The magnitude of I_{Na}-tail was proportional to increasing permethrin concentration (see also **Fig. 7** of **Lee et al.,** Mol Pharmacol 55:584-593).

3. The scorpion toxin LqhαIT synergizes permethrin: Vertebrate sodium channels are known to have distinct binding sites for various ligands, many of which interact allosterically. At least 6 binding sites are identified, including α-scorpion toxins (site 3), β-scorpion toxins (site 4) and pyrethroids (site 6) (**Catterall, 1992; Hayashi and Gard, 1994**). It is already known that different types of scorpion toxins may act cooperatively to produce enhanced insecticidal activity (**Lazarovici et al., 1982**). Also Wang and Strichartz (**1982**) reported a synergistic action between α- and β-scorpion toxins. This led us to consider the possibility that scorpion toxins may synergize other ligands, for example pyrethroids. Indeed, a recent report (**McCutchen et al., 1997**) suggests that a genetically engineered scorpion toxin enhances pyrethroid actions on *Heliothis virescens* larvae. Following construction of a dose-response curve for permethrin (**Fig. 2A, C**), then pre-exposed neurons to 200 pM LqhαIT and followed with application of various permethrin concentrations (**Fig. 2B**). **Fig. 2A** shows a typical concentration-dependent modification of sodium currents by permethrin, which is dramatically enhanced in the presence of 200 pM LqhαIT (**Fig. 2B, C**). Since the slopes of the concentration curves are dramatically different, we hypothesize that LqhαIT allosterically potentiates the action of permethrin, possibly by enhancing the binding of permethrin to sodium channels.

4. Sodium channels of pyrethroid-resistant insects are more sensitive to LqhαIT: We examined the possibility that sodium channels in pyrethroid-resistant *H. virescens* show changes in sensitivity to other ligands. LqhαIT dramatically slows inactivation of sodium channels in neurons of both susceptible and resistant insects (see **Lee et al.**, Mol Pharmacol, 55:584-593; **Fig. 8**) and interestingly, is more potent against pyrethroid-resistant sodium channels. To quantify modification of sodium currents in the two insect strains, we plotted elevation of steady-state sodium current {I_{Na} (s-s)} at various LqhαIT concentrations. The elevation of {I_{Na} (s-s)} was normalized to maximum elevation in each case. EC_{50} values were 0.71+0.03 and 1.84+0.06 nM in resistant and susceptible neurons. Slope factors (2.12+0.1 for

susceptible and 2.15+0.1 for resistant) were not different. According to these curves, resistant sodium channels were about 2.6 times more sensitive to this toxin. Our results are consistent with previous bioassays showing that 1) pyrethroid resistant strains of *H. virescens* may be more susceptible to genetically engineered baculoviruses containing a toxin gene than susceptible strains (**McCutchen et al., 1997**), and 2) kdr-resistant houseflies are more sensitive to a number of sodium channel ligands such as scorpion toxins than is the susceptible NAIDM strain (**Bloomquest and Miller, 1986**). This suggests that pyrethroid resistant insect strains might be dealt with using insect-selective peptide toxins in a manner such that resistance genes could be selected against.

5. Synergism between the scorpion toxin AaIT and pyrethroids: Genetically expressed scorpion toxins have been reported to show enhancement of pyrethroid toxicity against *H. virescens* (McCutchen, personal communication). It is likely that this positive cooperativity occurs at least in part at the level of the sodium channel, where distinct pharmacological binding sites are allosterically coupled. We found that Site 3 (alpha-scorpion toxin binding site; LqhαIT) and pyrethroid binding site show positive cooperativity. We extended this work by investigating the interaction between the excitatory scorpion toxin AaIT and permethrin.

We found that low concentrations of permethrin enhanced the action of AaIT against *H. virescens* sodium channels in central neurons. To understand the significance of this enhancement, it is necessary to digress slightly to explain the action of AaIT. We have found that AaIT shifts the voltage-dependence of sodium channel activation to more negative potentials (**Fig. 3A**). This shift only occurs at more negative potentials. When 10 nM permethrin is included in the bathing medium, much lower concentrations of AaIT are effective in shifting voltage-dependent activation (**Fig. 3B**). To further compare the effects of permethrin on channel modification by AaIT, we plotted the change in test depolarization potential (ΔV_T) for 50% channel activation vs. AaIT concentration (**Fig. 3C**). This plot clearly shows that the concentration dependence of this response is shifted to

lower AaIT concentrations by permethrin.

6. Sodium channels of Pyrethroid-resistant *H. virescens* are more sensitive to AaIT: Pyrethroid-R sodium channels also show enhanced sensitivity to the insect-selective excitatory scorpion toxin, AaIT. AaIT is known to shift voltage-dependent activation of insect sodium channels to more negative potentials, an effect we also observed in our experiments. We measured the peak sodium current as a function of test potential (V_T) at several toxin concentrations for susceptible (**Fig. 4A**) and pyrethroid-resistant budworms (**Fig. 4B**), then plotted the change in the test potential (ΔV_T) required to elicit 50% of maximal channel activation (**Fig. 4C**). At 50 nM AaIT, ΔV_T was 2.5+1 mV for UCR-S and 9.6+1.5 mV for Pyrethroid-R neurons, respectively. These data show that sodium channels in resistant neurons have a 4-fold higher sensitivity to AaIT than susceptible neurons.

7. LqhαIT slows sodium channel inactivation in housefly neurons: Data presented in a previous BARD report (1994) suggested that the molecular target of LqhαIT at the house fly neuromuscular junction is the sodium channel in presynaptic nerve membranes. To test this hypothesis, we examined the effects of these toxins on sodium channels in cultured house fly neurons. Since freshly dissociated neurons yielded very small or no sodium currents, a protocol was developed for short-term culture of house fly central neurons. All cultured neurons used in this study were dissociated from thoracic and abdominal ganglia of adult house flies. Robust, tetrodotoxin-sensitive voltage-dependent sodium currents were measured in cultured neurons with 1-2 days of short-term culture (**Fig. 5A**). Exposure of house fly neurons to LqhaIT (20 nM) resulted in a slowing of the fast inactivation of sodium currents, and increased the level of steady-state current (**Fig. 5A, B and C**). Voltage-dependent activation and peak sodium currents were not significantly affected (**Fig. 5D, 6A**). Modification of sodium channel inactivation by LqhαIT is particularly obvious in comparisons of steady-state inactivation, where LqhαIT (20 nM) shifted the h_∞ curve to more positive potentials (**Fig. 6B**). For example, under

normal conditions, a -20 mV prepulse inactivated virtually all available sodium channels in the absence of the toxin. Following LqhαIT treatment, more than 40% of the current remained at this prepulse potential. In addition, more than 10% of channels were not inactivated in the presence of the toxin at high positive potentials. LqhαIT did not change the voltage dependence of sodium channel activation (**Fig. 6A**). Test potentials to activate 50% of sodium channels, were -19.6+0.2 mV before and 20.8+0.6 mV after application of the toxin, respectively.

8. Sodium channels in pyrethroid-resistant houseflies: We compared the properties of susceptible vs. knockdown resistance (kdr) sodium channels, which have a point mutation (L1014F) in the II-S6 transmembrane segment. This mutation produces slight but significant changes in voltage-dependent gating properties. Voltage dependent activation is of kdr sodium channels is shifted 6.3 mV ($p< 0.05$) in the positive direction compared to the susceptible Cooper strain. Steady-state inactivation of kdr channels is shifted about 6.7 mV in the positive direction compared to Cooper (significantly different, $p<0.05$) (**Fig. 7A**). Sodium channels in kdr central neurons kdr sodium channels are less insensitive to permethrin compared to Cooper. Dose response curves showed that 1.2 mM permethrin modified 5% of sodium channels in Cooper, whereas 50 mM was necessary to modify 5% of channels in kdr (**Fig. 7B, C**).

9. Na^+ channels in resistant houseflies show no change in sensitivity to permethrin: Unlike sodium channels from pyrethoid-resistant *H. virescens*, sodium channels of housefly kdr neurons show no difference in sensitivity to the scorpion toxin LqhαIT (binds to pharmacological site 3) compared to the susceptible strain (**Fig. 8**).

Summary:

We provided a molecular basis for the physiological effects of LqhαIT previously observed. This toxin slows sodium channel inactivation without affecting voltage-

dependent activation. The slower inactivation rate leads to broadening of the action potential. Prolonged depolarization at the nerve terminal will cause a longer period of calcium entry and increased neurotransmitter release.

We established pharmacological profiles for sodium channels in the pest insect *H. virescens*. Molecular actions of LqhαIT and permethrin were intensively studied at the level of macroscopic sodium currents. We showed that LqhαIT synergizes permethrin action against sodium channels, and that pyrethroid-resistant sodium channels are more sensitive to pyrethroids. We have found that both Site 3, as defined by LqhαIT binding, and the AaIT binding site show positive cooperativity with the pyrethroid binding site on the *H. virescens* sodium channel.

Figure Legends:

Fig. 1. Effects of LqhαIT on sodium channels in cultured *H. virescens* neurons. (A) Control sodium current family. LqhαIT (40 nM) dramatically slowed sodium channel inactivation and peak sodium currents (B). Steady-state sodium currents {I_{Na}(late)} indicated by down-arrows in (A) and (B) were plotted as a function of test potentials before and after application of LqhαIT (C). I_{Na}(late) was dramatically elevated in a voltage-dependent manner. Also current-voltage relationship was expressed in the plot (D) before and after application of LqhαIT. The toxin increased peak sodium currents. Holding potential was -100 mV. (E-F) Voltage-dependent activation and steady-state inactivation of sodium channels. Voltage-dependent activation of channels was expressed by normalized conductance and plotted as a function of test potentials (A). Sodium conductance was calculated by a equation; $g_{Na} = I_{Na} / (V_T - E_{rev})$, where g_{Na} is sodium conductance and I_{Na} is a peak current evoked by a test potential (V_T) while E_{rev} is the reversal potential of sodium channels. LqhαIT did not modify voltage dependency of sodium channels. (F) Steady-state inactivation of sodium channels was plotted using the traditional double pulse protocol comprising prepulses and the test potential (inset). Each peak sodium current was evoked by test potential (V_T) following various prepulses. Then peak sodium currents were normalized to the maximum peak sodium current

and plotted as a function of prepulses. LqhαIT dramatically inhibited steady-state inactivation. For example, all sodium channels were inactivated at -5 mV prepulse before toxin treatment while ~70% of sodium channels were not inactivated at the same prepulse after application of the toxin.

Fig. 2. Potentiation of permethrin action by 200 pM LqhαIT. (A) Sodium currents {I_{Na}(s-s tail)} were modified in a concentration-dependent manner by permethrin. (B) Pre-exposure to 200 pM LqhαIT dramatically potentiated permethrin action. Numbers in (A) and (B) are permethrin concentrations. (C) Concentration-response curve for permethrin alone (circles) or permethrin following pre-treatment with 200 pM LqhαIT (triangles). The elevation of I_{Na}(s-s tail) was normalized to the control peak sodium current and plotted as shown in Fig. 3. Bars are SEM's.

Fig. 3. Enhancement of AaIT action by permethrin. A) Application of 100 nM AaIT causes a doubling of peak current sodium current recorded from a *H. virescens* central neuron. However, in the presence of 10 nM permethrin, peak current in the same concentration of AaIT is doubled yet again. B) Current-voltage relationship of sodium current recorded under control conditions, 100 nM AaIT, and 100 nM AaIT in the presence of 10 nM permethrin. Note the shift of the activation curve to the left relative to control. C) Normalized sodium channel conductance (gNa) plotted as a function of test potential (V_T). 100 nM AaIT shifts voltage-dependent activation to the left. In the presence of 10 nM permethrin, this shift is much more pronounced.

Fig. 4. Differential sensitivity of sodium currents in UCR-S and Pyrethroid-R neurons to AaIT. Upper left: representative gNa/V curves for control and three AaIT concentrations tested on UCR-S neurons: 25, 50, and 100 nM. Right: same experiment performed on Pyrethroid-R neurons. (C) ΔV_T to activate 50% of channels was plotted as a function of AaIT concentration for UCR-S and Pyrethroid-R neurons; sodium channels in resistant *H. virescens* showed 4-fold higher sensitivity to the toxin at 50 nM AaIT.

Fig. 5. Effects of LqhαIT (20 nM) on sodium channels in the house fly neurons. Sodium current families before (A) and after (B) application of LqhαIT evoked by

test potentials from -45 to -10 mV with 5 mV increment. LqhαIT caused a slight increase in peak sodium current and a slowing of sodium channel inactivation. Holding potential was -105 mV. (C) Steady-state sodium currents were taken at points indicated in (A) and (B), and plotted as a function of test potentials. (D) Typical I/V curves before and after application of LqhαIT. Peak sodium currents were plotted against test potentials.

Fig. 6. Voltage-dependent activation (A) and inactivation (B) of sodium channels in house fly neurons before and after LqhαIT application (20 nM). (A) Normalized sodium conductance was plotted as a function of test potentials. Each value for sodium conductance (gNa) was calculated by an equation, $g_{Na}=I_{Na}/(V_T - E_{rev})$ and normalized to maximum sodium conductance. I_{Na} is a peak current evoked by a test potential (V_T) and E_{rev} is the reversal potential of sodium current. LqhαIT did not change voltage dependence of sodium channels. (B) A double-pulse protocol (inset; see Text) was used to plot sodium channel inactivation curves. Each peak sodium current was evoked by a test potential (V_T; -10mV) following various prepulses (V_P). Holding potential was -105 mV. Peak sodium currents were normalized to the maximum peak sodium current and plotted as a function of prepulses. As can be seen in (B), LqhαIT inhibited inactivation of sodium channels. Bars indicate SEMs in (A) and (B).

Fig. 7. Modification of Cooper (susceptible) and kdr houseflies by permethrin. (A) Permethrin causes visible prolongation of tail currents beginning at 60 nM, and shows concentration-dependent modification up to 3 mM. (B) When tested on kdr neurons, no effects of permethrin are observed below 30 mM. (C) Percent modification of channels as a function of permethrin concentration.

Fig. 8. No difference in sensitivity to LqhαIT is observed when Cooper sodium channels are compared to those of kdr.

Description of the cooperation

The mode of action at the channel site and synergistic actions of LqhαIT have been performed with the recombinant toxin obtained and purified in Tel-Aviv. Two mutants (R64H and K62R) displaying some elevated toxicity and binding affinity to the receptor binding site were analyzed in Riverside at the channel site (see results).

The Riverside laboratory studied the cooperative effects of recombinant LqhαIT and the excitatory toxin AaIT on pyrethroid application to the channel. The data obtained together with recent reports by McCutchen et al., **1997**, strongly suggest that a drop in the amounts of pyrethroids applied in the field is obtainable without loss of effect due to the synergistic effects produced by the application of baculoviruses expressing these toxins.

Most recently, the Riverside laboratory is on way to establish a functional recombinant sodium channel in oocytes. This system will be used extensively to study our toxin mutants *vis a vis* mutagenesis of the channel in order to elucidate the interacting surface. It is definitely the joint project, which enabled this stage to arrive and hopefully be fruitfull in the next years.

Both PIs have visited the laboratories of their collaborators to discuss the joint work (particularly of students from both sides; O. Froy and M. Turkov at Tel-Aviv, and T. Norris and D. Lee at Riverside), and to write joint papers.

The cooperation between both laboratories yielded thus far several joint papers:

Zlotkin E, Gurevitz M, Fowler E, Moyer M, Adams ME (1993) Depressant insect Selective neurotoxins from scorpion venom: chemistry, action and gene cloning. Arch Insect Biochem Physiol, 22:55-73.

Zilberberg N, Gordon D, Pelhate M, Adams ME, Norris T, Zlotkin E, Gurevitz M (1996) Functional expression and genetic alteration of an alpha scorpion neurotoxin. Biochemistry, 35:10215-10222.

Gurevitz M, Froy O, Zilberberg N, Turkov M, Strugatsky D, Gershburg E, Lee D, Adams ME, Tugarinov V, Anglister J, Shaanan B, Loret E, Stankiewicz M, Pelhate M, Gordon D, Chejanovsky N (1998) Sodium channel modifiers from scorpion venom: Structure-activity relationship, mode of action, and application. Toxicon, 36:1671-1682.

Gurevitz M, Froy O, Zilberberg N, Shaanan B, Adams ME, Anglister J, Pelhate M, Chejanovsky N, Gordon D (1999) Sodium channel modulators from scorpion

venoms: Structure-activity relationship and application. Neurotox 98, Oxford, UK. In press.
Lee, D., Gurevitz, M., and Adams, M.E. Synergism between insect selective scorpion toxins and pyrethroid insecticides. To be submitted.
Lee, M. Gurevitz, and M.E. Adams. Modification of neuromuscular transmission and insect sodium channels by insect-selective scorpion toxin LqhαIT. To be submitted.

Other non joint papers produced during BARD support are described below.

Evaluation of the research achievements

The structure-activity studies conducted using point mutagenesis followed by biological assays of all toxin variants, and the determination of the 3-D structure of LqhαIT by NMR, enabled us to determine the toxic surface of the toxin. Recent, yet unpublished, mutants including chimeras aimimg at a reciprocal exchange of active site with a mammalian alpha toxin (LqhαIT x Lqh II), have clearly established the toxic site and boundaries (**unpublished**). This fulfils our first objective.

The other objectives were to clarify the mode of action at the channel site and synergism between scorpion toxins and insecticidal chemicals. This study was carried out successfully in Riverside and is in the process of getting published. The implications of such findings can now be examined for their practical significance using baculoviruses and pyrethroids.

It is therefore concluded that the original objectives of this research have been met quite successfully.

Conclusions

The joint project on scorpion insecticidal neurotoxins and their receptors sites on sodium channels is attractive from both academic point of view, e.g. structure-activity relationship, protein folding, ion channel gating, and applicative side. It is for a number of years that both laboratories work on this project, which yielded the following results:

1) The genes for the toxins have been cloned and expressed using an efficient bacterial expression system. This enabled analysis of the toxins via a genetic

dissection and detrmining the 3-D structure of several representatives. Such information will eventually lead to the synthesis of improved, selective insect killers and perhaps toxin mimetics.

2) The cDNA clones of several toxins have been expressed in insect cells and larvae using baculoviral vectors. This approach provided a direct means for crop protection, which replaces current pesticides, or may reduce their hazardous overuse.

3) The mode of action of scorpion toxins at the ion channel level was clarified and shown to interact cooperativly with conventional insecticides. This is an important contribution to the future utilization of pest control agents, which are more friendly to the environment and human health. This study also initiates a better understanding of the channel action, which will be exploited to determine a valueable data regarding the toxins' receptor binding sites.

4) Similar approaches have been undertaken in the current BARD supported project using toxins, which are totally selective for insect sodium channels (excitatory and depressant toxins). We expect to provide during next years the coordinates of several bioactive surfaces of anti-insect specifc killers and gain information on their receptor binding sites. Such information is is of great value for future pest control as well as the academic facet of ligand-receptor interactions.

The data produced during this research period provide a further rationale for using combinations of scorpion toxins and pyrethroids for more efficient insect control. Two final conclusions with potential for practical application emerge from this work:

1. A combined application of insect-selective peptide toxins (via baculoviruses) with pyrethroids may allow a lower rate of pyrethroid application in the field without sacrificing efficacy.

2. The enhanced potency of LqhαIT against sodium channels of resistant insects suggests a means of diluting the frequency of gene(s) conferring pyrethoid resistance in pest populations.

Publications during BARD support

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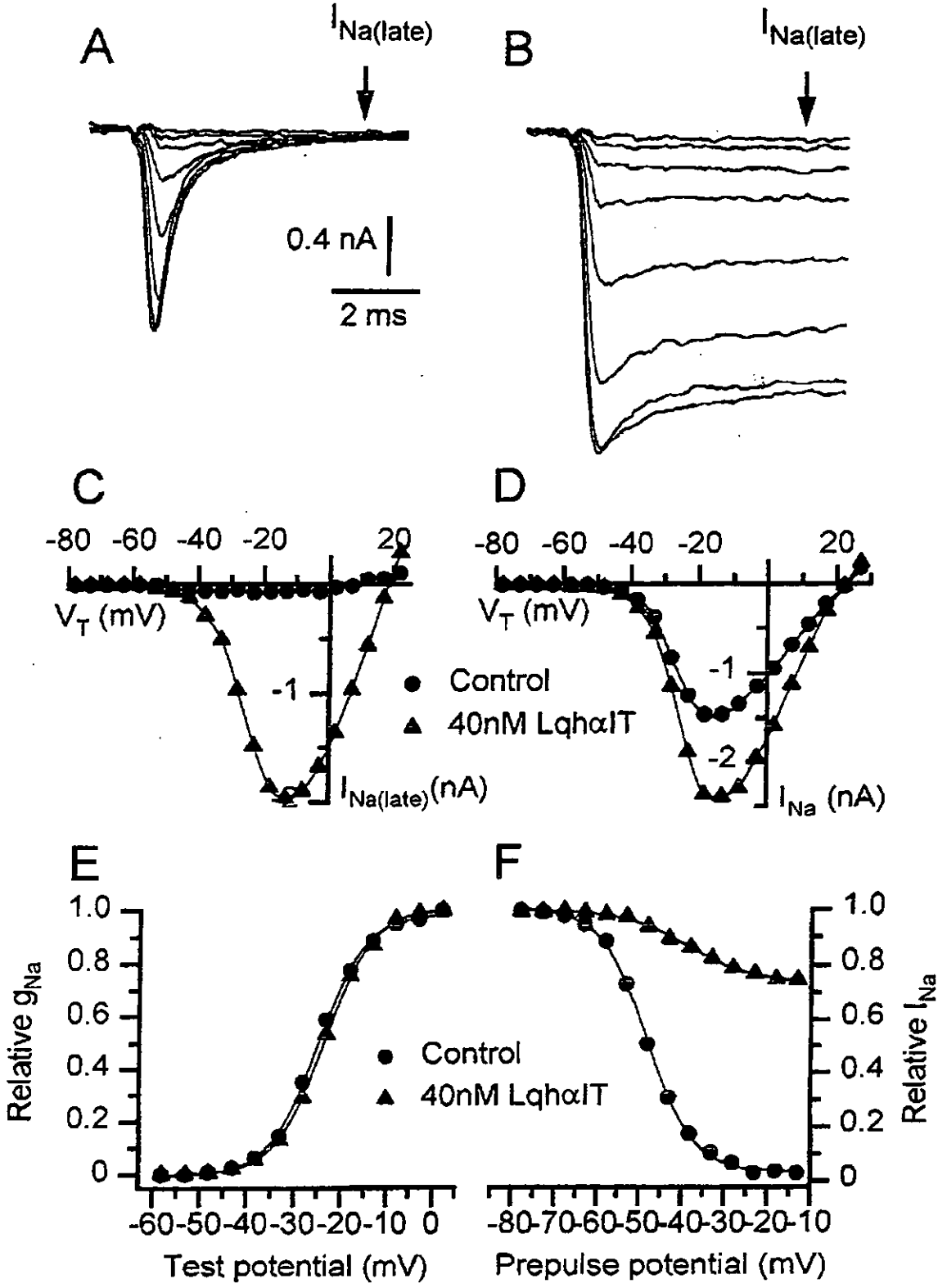

A
$I_{Na(late)}$
0.4 nA
2 ms
B
$I_{Na(late)}$
C
-80 -60 -40 -20 20
V_T (mV)
-1
Control
40nM LqhαIT
$I_{Na(late)}$(nA)
D
-80 -60 -40 -20 20
V_T (mV)
-1
-2
I_{Na} (nA)
E
Relative g_{Na}
1.0
0.8
0.6
0.4
0.2
0
Control
40nM LqhαIT
-60-50-40-30-20-10 0
Test potential (mV)
F
Relative I_{Na}
-80-70-60-50-40-30-20-10
Prepulse potential (mV)

Fig. 1

Fig. 2

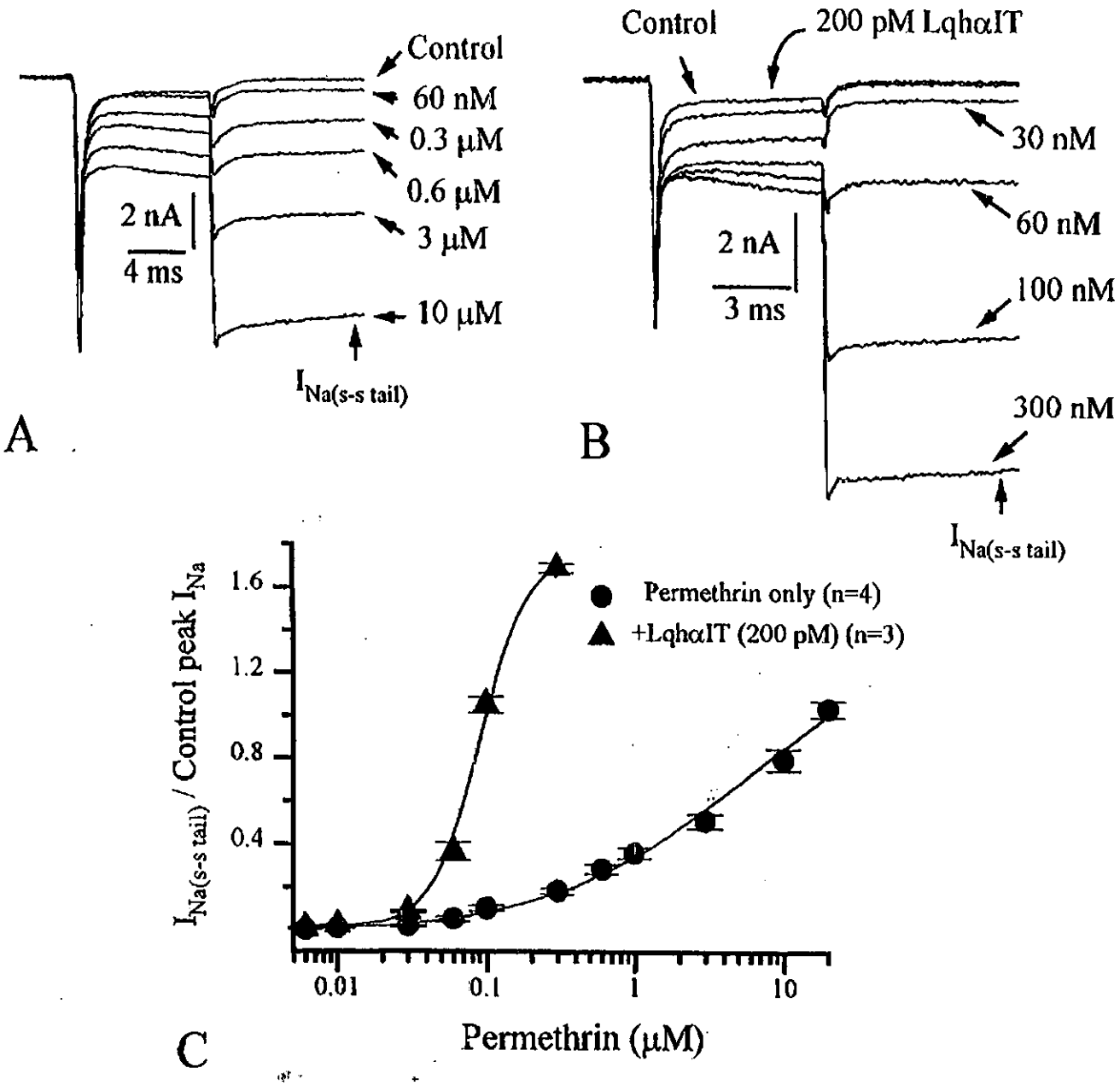
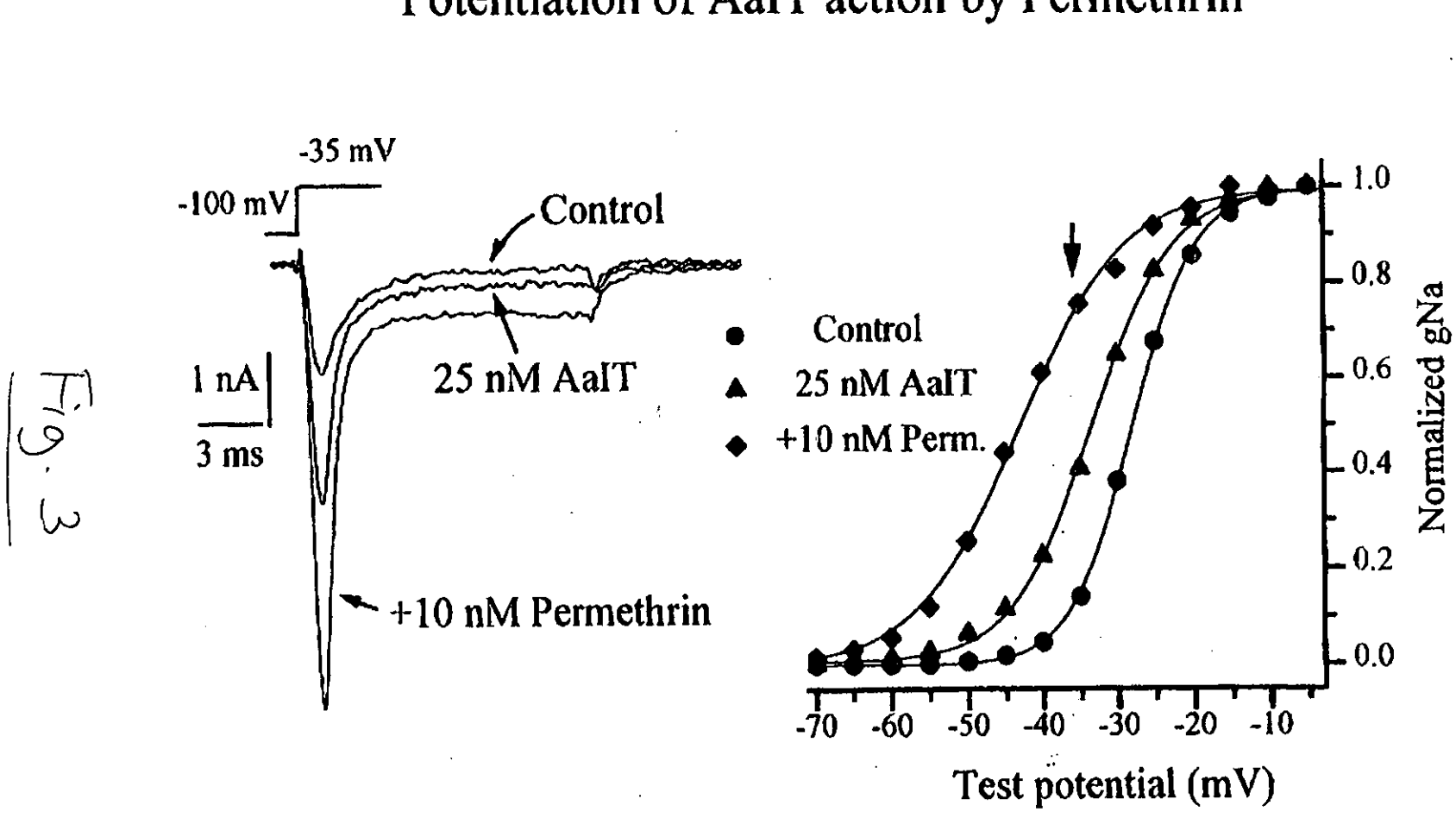

Control
60 nM
0.3 μM
0.6 μM
3 μM
10 μM
2 nA
4 ms
$I_{Na(s\text{-}s\ tail)}$
A
Control
200 pM LqhαIT
30 nM
60 nM
100 nM
300 nM
2 nA
3 ms
$I_{Na(s\text{-}s\ tail)}$
B
Permethrin only (n=4)
+LqhαIT (200 pM) (n=3)
$I_{Na(s\text{-}s\ tail)}$ / Control peak I_{Na}
1.6
1.2
0.8
0.4
0.01
0.1
1
10
Permethrin (μM)
C

Fig. 3


Potentiation of AaIT action by Permethrin
-35 mV
-100 mV
Control
25 nM AaIT
1 nA
3 ms
+10 nM Permethrin
Control
25 nM AaIT
+10 nM Perm.
1.0
0.8
0.6
0.4
0.2
0.0
Normalized gNa
-70
-60
-50
-40
-30
-20
-10
Test potential (mV)

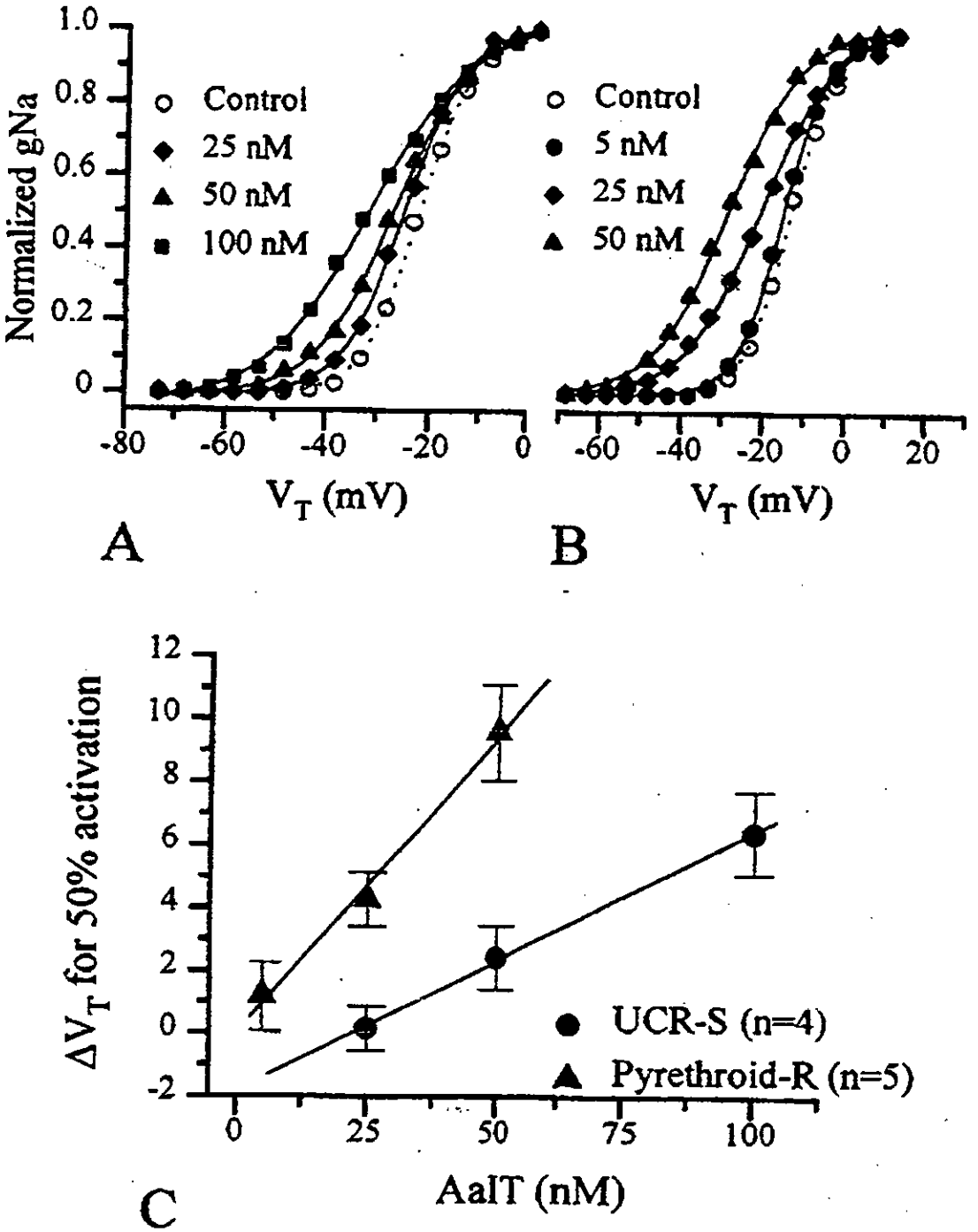

Normalized gNa
1.0
0.8
0.6
0.4
0.2
0
Control
25 nM
50 nM
100 nM
-80
-60
-40
-20
0
V_T (mV)
A
Control
5 nM
25 nM
50 nM
-60
-40
-20
0
20
V_T (mV)
B
ΔV_T for 50% activation
12
10
8
6
4
2
0
-2
UCR-S (n=4)
Pyrethroid-R (n=5)
0
25
50
75
100
AaIT (nM)
C

Fig. 4

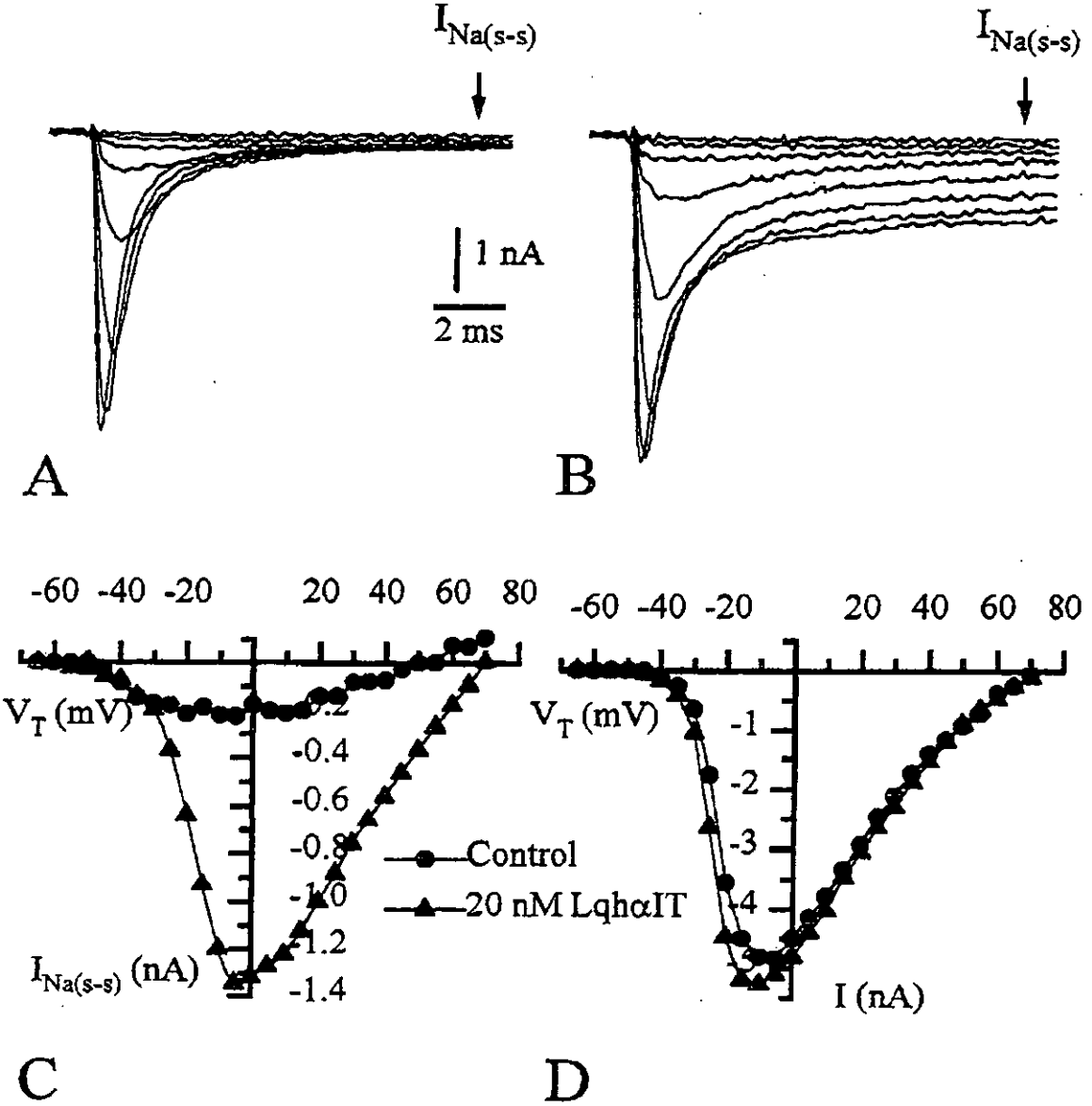

$I_{Na(s-s)}$
1 nA
2 ms
A
$I_{Na(s-s)}$
B
-60 -40 -20 20 40 60 80
V_T (mV)
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
Control
20 nM LqhαIT
$I_{Na(s-s)}$ (nA)
C
-60 -40 -20 20 40 60 80
V_T (mV)
-1
-2
-3
-4
I (nA)
D

Fig. 5

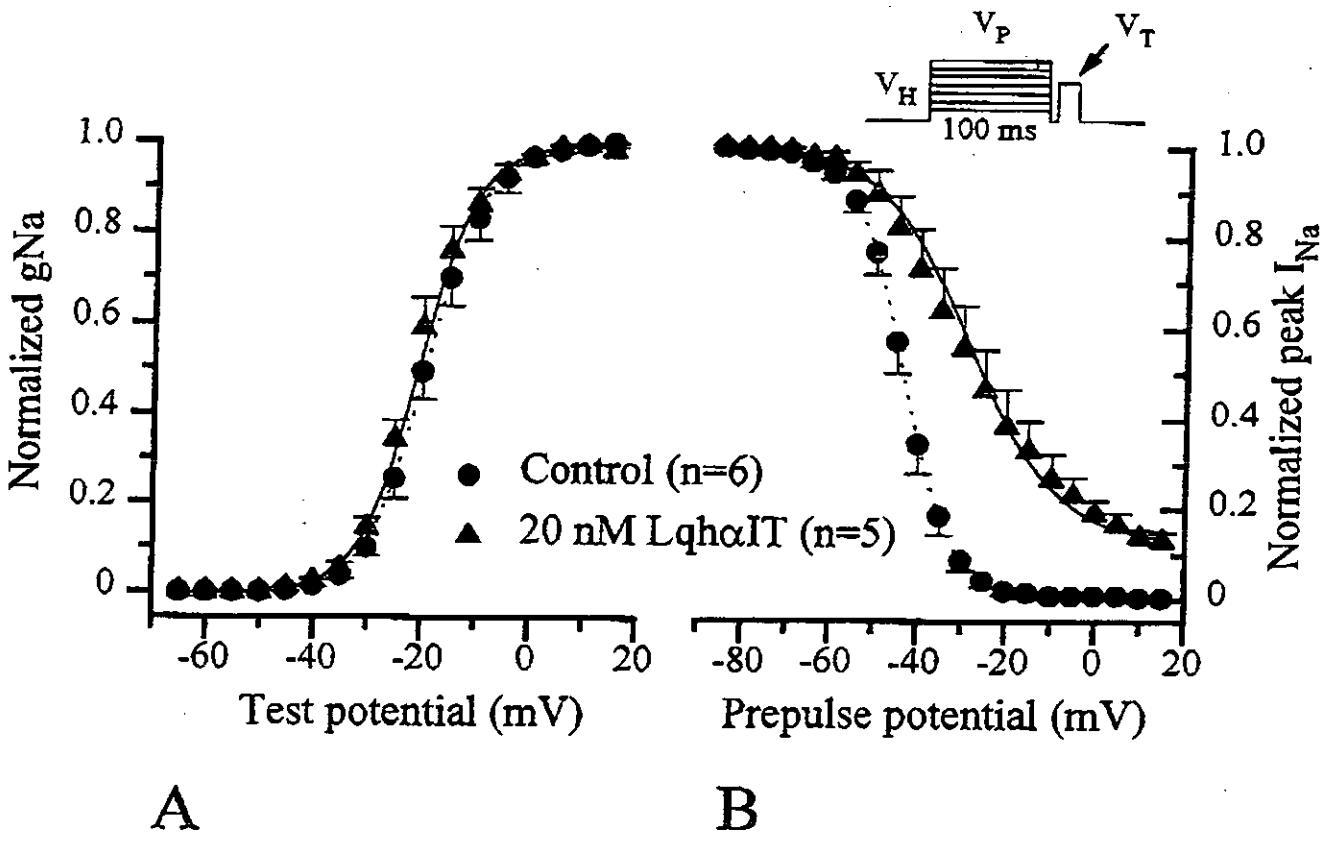
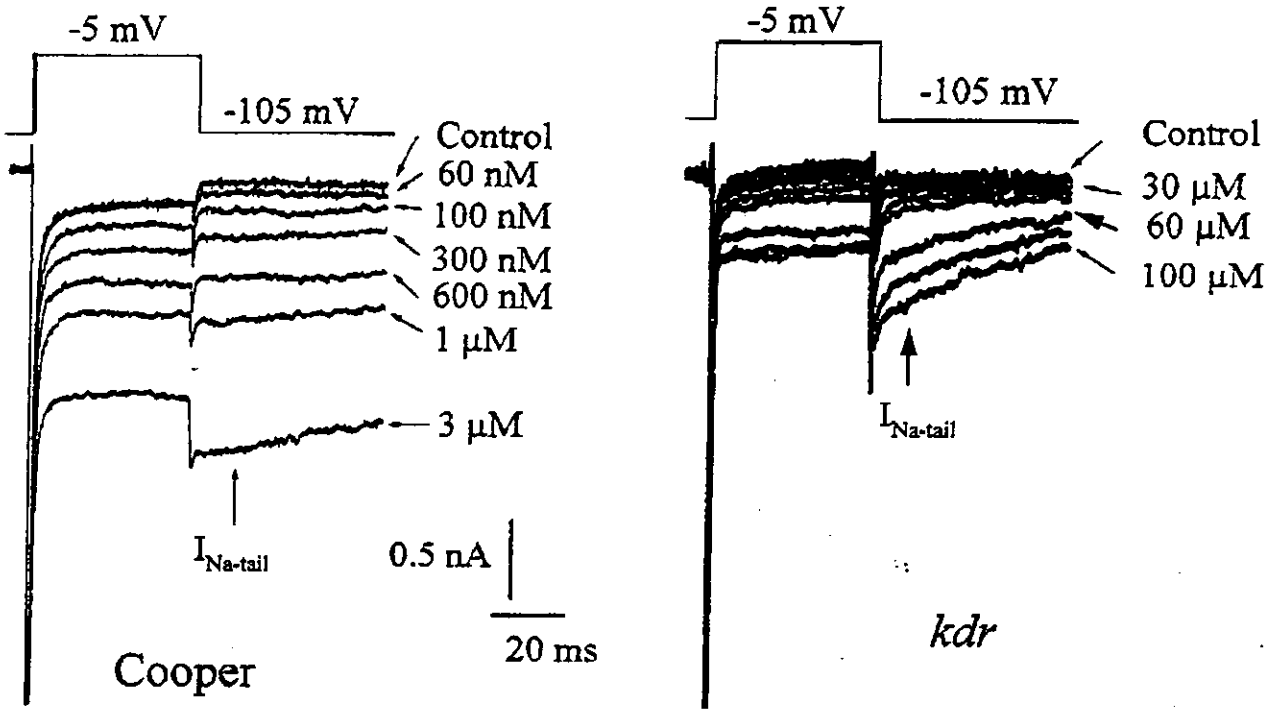

Normalized gNa
1.0
0.8
0.6
0.4
0.2
0
-60
-40
-20
0
20
Test potential (mV)
Control (n=6)
20 nM LqhαIT (n=5)
A
V_P
V_T
V_H
100 ms
Normalized peak I_{Na}
-80
-60
-40
-20
0
20
Prepulse potential (mV)
B

Fig. 6


-5 mV
-105 mV
Control
60 nM
100 nM
300 nM
600 nM
1 μM
3 μM
$I_{Na\text{-}tail}$
0.5 nA
20 ms
Cooper
A
-5 mV
-105 mV
Control
30 μM
60 μM
100 μM
$I_{Na\text{-}tail}$
kdr
B

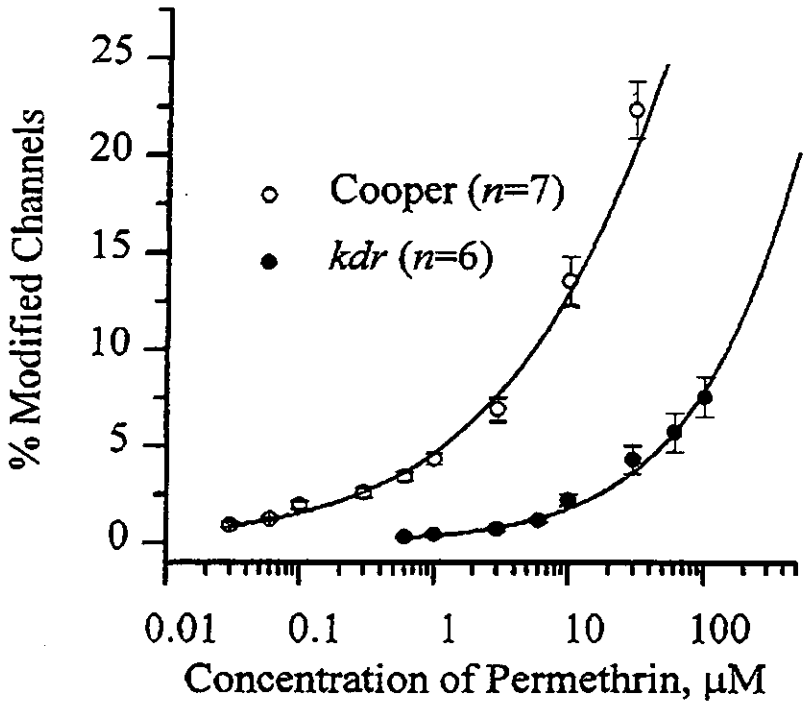

% Modified Channels
25
20
15
10
5
0
Cooper (n=7)
kdr (n=6)
0.01
0.1
1
10
100
Concentration of Permethrin, μM
C

Fig. 7

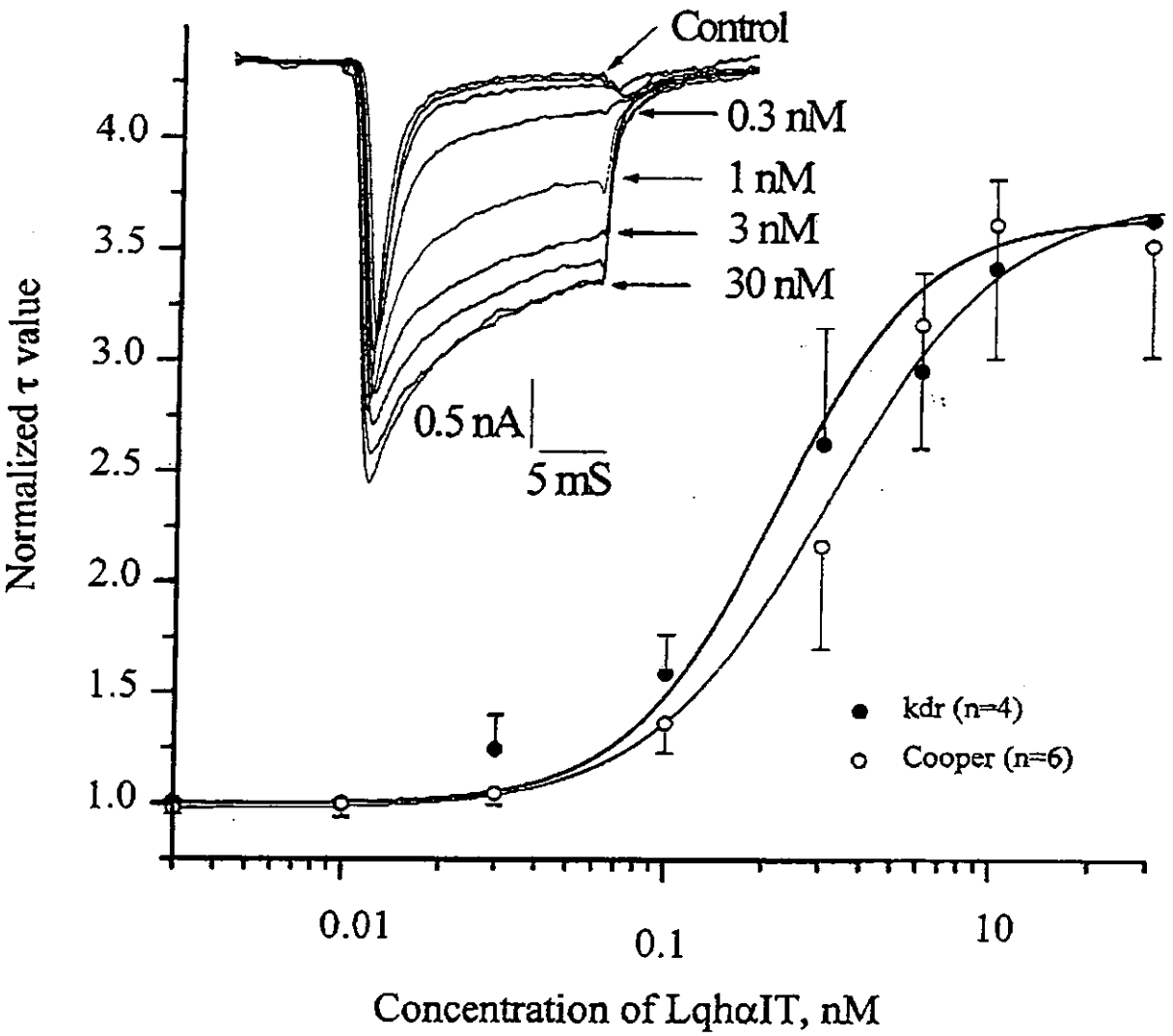

Control
0.3 nM
1 nM
3 nM
30 nM
0.5 nA
5 mS
Normalized τ value
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.01
0.1
10
kdr (n=4)
Cooper (n=6)
Concentration of LqhαIT, nM

Fig. 8